SEED PHYSIOLOGY & METABOLISM

Elevated Temperature and Carbon Dioxide Effects on Soybean Seed Composition and Transcript Abundance

J. M. G. Thomas,* K. J. Boote, L. H. Allen, Jr., M. Gallo-Meagher, and J. M. Davis

ABSTRACT

Climate change due to increased [CO_2] and elevated temperature may impact the composition of crop seed. This study was conducted to determine the potential effects of climate change on composition and gene expression of soybean [*Glycine max* (L.) Merr. cv. 'Bragg'] seed. Soybean plants were grown in sunlit, controlled environment chambers under diel, sinusoidal temperatures of 28/18, 32/22, 36/26, 40/30, and 44/34°C (day/night, maximum/minimum), and two levels of [CO_2], 350 and 700 μmol mol^{-1}, imposed during the entire life cycle. The effect of temperature on mature seed composition and transcripts in developing seed was pronounced, but there was no effect of [CO_2]. Total oil concentration was highest at 32/22°C and decreased with further increase in temperature. Oleic acid concentration increased with increasing temperature whereas linolenic acid decreased. Concentrations of N and P increased with temperature to 40/30°C, then decreased. Total nonstructural carbohydrates (TNC) decreased as temperatures increased, and the proportion of soluble sugars to starch decreased. Transcripts of a gene that is downregulated by auxin (ADR12) were dramatically downregulated by elevated temperature, possibly reflecting the altered course of seed development under environmental stress. Transcripts of β-glucosidase, a gene expressed during normal soybean seed development, were detected in seed grown at 28/18°C but not in seed grown at 40/30°C, which also suggests that normal programs affecting seed composition were perturbed by elevated temperature. These results confirm previous studies indicating that high temperature alters soybean seed composition, and suggest possible mechanisms by which climate change may affect soybean seed development and composition.

The [CO_2] in our atmosphere has risen sharply during the last 50 yr, as shown by records obtained at Mauna Loa, HI (Keeling et al., 1995). Elevated temperatures are expected to accompany the increased [CO_2] because of the additional greenhouse effect of this gas in the atmosphere (Intergovernmental Panel on Climate Change, 1995). Potential impacts of such climate changes on grain crop species should be determined, especially on crops like soybean that are essential to the world's food supply. Curry et al. (1995) predicted decreased soybean yields in the southeastern USA associated with a 5°C increase in temperature predicted by several global climate change models. Allen and Boote (2000) reviewed the documented impacts of climate change on soybean, including decreased yield and quality due to higher growth temperature. Sunlit, controlled environment chambers have been used to examine the effects and interactions of increased [CO_2] and temperature on plants (Pickering et al., 1994), revealing important alterations of physiology, growth, and seed yield (Baker and Allen, 1993; Allen and Boote, 2000).

The unique chemical composition of soybean has made it one of the most valuable agronomic crops worldwide. Consumed for thousands of years in Asia, soybean has steadily gained importance as food in the USA, and many new products have been developed using soybean seed as raw material (Liu, 1997). Oil and protein comprise ≈20 and 40%, respectively, of the dry weight of soybean seed, and approximate values of other components include carbohydrates (30%), crude fiber (5%), and ash (5%) (Hymowitz et al., 1972). In addition, soybean contains minerals such as Fe, Cu, Mn, Ca, Mg, Zn, Co, P, and K. Vitamins B_1, B_2, and B_6, as well as isoflavones, are also available in soybean (Augustin and Klein, 1985; Messina, 1997).

The intrinsic value of soybean seed is in its supply of essential fatty acids and amino acids in the oil and protein, respectively. Soybean seed composition is affected by both genetic and environmental variables, as well as the interaction of the two (Honeycutt et al., 1989; Piper and Boote, 1999). Several studies have shown changes in seed composition due to growth temperatures ranging from 15/12 to 40/30°C, but none of these studies included high [CO_2] (Wolf et al., 1982; Carver et al., 1986; Rennie and Tanner, 1989; Dornbos and Mullen, 1992; Gibson and Mullen, 1996; Rebetzke et al., 1996). As growth temperature of field-grown soybean increased up to a mean of 28°C, oil concentration increased. Protein concentration decreased from 14°C to a minimum at 22°C, at which point it began to increase with temperature up to 28°C (Piper and Boote, 1999). In addition to changes in the concentration of oil produced in seed, the ratio of fatty acids in soybean oil changes when seeds develop under elevated temperature. For example, oleic acid concentration increased with increasing temperatures while linoleic acid decreased (Carver et al., 1986; Rennie and Tanner, 1989; Gibson and Mullen, 1996; Rebetzke

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Abbreviations: DAT, days after tagging; DD, differential display; EST, expressed sequence tag; FAME, fatty acid methyl ester; GC, gas chromatograph; TNC, total nonstructural carbohydrates.

et al., 1996). Heagle et al. (1998) observed a positive, significant effect of CO_2 enrichment on soybean seed oil and oleic acid concentration, especially when plants were grown under elevated O_3.

Presumably, high temperature or CO_2–induced changes in seed composition are mediated at the level of gene expression. In soybean, however, studies on temperature or [CO_2] effects on seed have not yet been associated with the expression of specific genes related to seed storage compounds. The ratio of polyunsaturated to monounsaturated fatty acids in soybean oil is known to decrease with high temperature (Wolf et al., 1982; Rebetzke et al., 1996); however, the abundance of transcripts encoding fatty acid desaturase did not change (Heppard et al., 1996). Liang and Pardee (1992) developed differential display (DD) of messenger RNA, a two-dimensional RNA fingerprinting method that is suited to identifying inducible genes. Sequences of such genes could provide information on the types of cellular processes that are changed as a consequence of environmental changes. From a seed biology standpoint, identifying differentially regulated genes can provide insights into how seeds respond to stress.

Few studies have been conducted to specifically document changes in seed composition caused by climate change, that is, elevated [CO_2] and higher temperature. The focus of this study was on temperatures above 28/18°C, which could occur because of global warming. Vegetative growth processes and seed production of the cultivar 'Bragg' document that elevated temperature treatments influenced the yield and decreased the weight per seed (Baker et al., 1989). In the present paper, composition was determined for seed grown at a wide range of temperatures from 28/18 to 44/34°C (day/night) in the sunlit, controlled environment chamber experiments conducted by Pan (1996). Knowledge of the chemical composition of these seed should aid in ascertaining how environmental parameters affect the nutritional value of soybean seed. The first objective of this work was to examine the composition of mature soybean seed produced on plants grown at two levels of [CO_2], 350 and 700 μmol mol^{-1}, and temperatures of 28/18, 32/22, 36/26, 40/30, and 44/34°C (day/night, maximum/minimum). The second objective was to determine if differences in transcript abundance were evident in immature seed that could influence developmental programs that affect seed composition.

MATERIALS AND METHODS

Plant Material and Experimental Treatments

Soybean (cv. Bragg) was grown in two experiments in eight sunlit, controlled environment chambers (Pickering et al., 1994) under two levels of [CO_2], 350 and 700 μmol mol^{-1}. Temperature treatments were imposed during the entire plant life cycle in a diel, sinusoidal control pattern. During the first experiment (Exp. 1), sown on 19 Aug. 1993, temperatures were 28/18, 32/22, 36/26, and 40/30°C (day/night, maximum/minimum) and the experiment was designed as a balanced two-[CO_2] by four-temperature factorial.

In the second experiment (Exp. 2), sown on 11 Feb. 1994, an increased range of temperatures was imposed to ensure that the point of reproductive failure would be reached. During Exp. 2, temperatures for plants under 700 μmol CO_2 mol^{-1} air were 28/18, 32/22, 36/26, 40/30, 44/34, and 48/38°C and plants at 350 μmol CO_2 mol^{-1} were grown at only two temperatures, 28/18 and 40/30°C. Daylength in both studies was the prevailing natural cycle for Gainesville, FL. The effects on composition of seeds grown at 700 μmol CO_2 mol^{-1} air under five temperatures were analyzed by regression.

Standard cultural practices were followed as described by Pan (1996). Seed were inoculated with *Bradyrhizobium japonicum* before sowing in Kendrick fine sand (loamy, siliceous, hyperthermic Arenic Paleudults) topsoil that was 0.55 m deep. Magnesium sulfate and fenamiphos [15 g ethyl 3-methyl-4-(methylthio)phenyl (1-methylethyl)phosphoramidate] were applied at the rates of 34 g m^{-2} and 4.48 g m^{-2}, respectively, and incorporated into the top 15 cm of soil. Six rows at 32-cm row spacing were established in each 1-m-wide by 2-m-long by 1.5-m-high chamber. Plants were thinned to 75 plants m^{-2} at 11 d after planting (DAP). No fertilizer N was applied and most roots were well-nodulated, indicating that the inoculation with N_2 fixing *B. japonicum* was successful. Plants were sampled during the course of the growing season for growth analyses reported elsewhere (Pan, 1996), and as a result of that sampling there were 35 and 21 plants m^{-2} in Exp. 1 and Exp. 2, respectively, at the final harvest.

Seed Sampling

Composition Analysis

Seed were collected at harvest maturity. The actual harvest dates varied according to the temperature treatment, and are reported in Thomas (2001). Plants grown at 44/34°C were not yet mature at harvest and produced only a few seed, which were included in the composition analyses.

Pods from final harvest samples were dried with forced air at ≈23°C for 1 wk. Seed were then removed from the pods. Measured moisture content of seed was between 6 and 8%. Seed were stored dry for about one month before preparing samples for composition analysis. Then, three 100-seed samples were taken from each treatment. The seed were ground first in a single rotary blade grinder manufactured by IKA WERK[1] (Staufen, Germany) and then milled to a fine powder in a WARING (Torrington, CT) blender to achieve a uniform particle size. The ground seed samples were stored at −20°C until composition analyses were conducted.

Transcript Analysis

Pods in the first experiment were tagged at the R3 stage (≈5 mm in length) (Fehr and Caviness, 1977) for subsequent growth sampling. Seed growth was delayed at 40/30°C compared with 28/18°C. Thus, the pods at 40/30°C were tagged later, and reporting the age as days after tagging (DAT) adjusts for this delay (Fig. 1). The fresh weight of each seed was measured immediately after removal from the pod. Each seed was then dissected, separating the testa, embryonic axis, and cotyledons. The embryonic axes and cotyledons from each pod were frozen in liquid N_2 in separate 1.5-mL plastic microcentrifuge tubes, and stored at −80°C until RNA extraction.

Immature seed samples were taken from low and high temperature treatments, 28/18 and 40/30°C, at 68 or 85 DAP, corresponding to 26 or 30 DAT, respectively (Thomas, 2001).

[1] Mention of proprietary products is for the convenience of the reader only, and does not constitute endorsement or preferential treatment by USDA-ARS or the University of Florida.

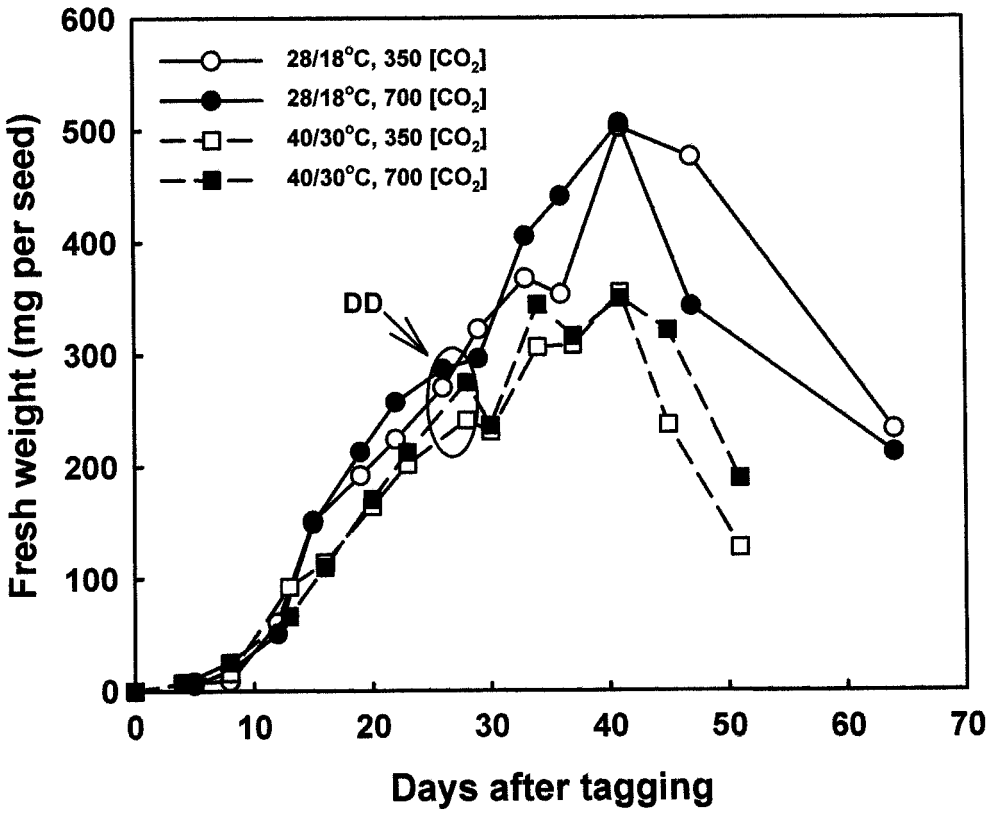

Fig. 1. Soybean seed fresh weight vs. days after tagging in Exp. 1 at 350 or 700 μmol mol^{-1} [CO_2]. The ellipse encircles the samples from 28/18 and 40/30°C, respectively, used for differential display (DD). In contrast, seed tested for composition had reached full maturity and had achieved maximum dry weight.

There was no effect of [CO_2] level on maturity; thus seed of different [CO_2] treatments were sampled on the same dates within temperature treatments. Criteria of similar fresh weight and DAT were used to select seeds of approximately similar developmental stage for testing DD in each temperature treatment (Liang and Pardee, 1992). Seed used for DD of messenger RNA weighed between 231 and 287 mg fresh weight. Samples for DD were either 26 DAT (20 d before R7) for 28/18°C, or 30 DAT (20 d before R7) for 40/30°C treatments (Fig. 1).

Lipid and Fatty Acid Analysis

To determine the concentration of soybean oil, the extraction method of Sasser (1990) was used and lipids were extracted from each 100-seed sample two times. Each time, three 1-g samples of ground seed from each test chamber were weighed into preweighed test tubes and covered with 5 mL of hexane:methyl tert-butyl ether solvent (1:1 v:v). The mixture was vortexed for 2 min and left overnight at 25°C. The tubes were vortexed again and then centrifuged for 7 min at 1300 rpm (≈12 000 × *g*). The solvent was removed with a Pasteur pipette and 5 mL solvent was added to the partially defatted powder. The process of vortexing and centrifuging was repeated for a second 6-h extraction. The solvent was evaporated under N_2 at 45°C and total oil was determined gravimetrically.

Seed oil obtained from the total oil analysis was prepared for fatty acid analysis. The preparation of fatty acid methyl esters (FAME) was based on the method of Maxwell and Marmer (1983). Approximately 20 mg of lipid (1 drop), extracted from ground seed with hexane:methyl tert-butyl ether solvent (1:1 v:v), was added to a leak-proof, Teflon-lined screw cap tube and 2 mL of isooctane were added. Then, 100 μL of 2 *M* KOH in methanol (0.11 g mL^{-1}) were added, the tube was vortexed for 1 min and then centrifuged at 12 000 × *g* for 5 min. The lower methanol layer was discarded and 0.5 mL of saturated ammonium acetate was added; the tube was vortexed 1 min then centrifuged at 12 000 × *g* for 5 min, and the lower layer was discarded. The procedure was repeated using water instead of ammonium acetate. About 100 mg of sodium sulfate was added, the mixture was held at 25°C for 5 min, and then centrifuged (12 000 × *g*). The top layer was placed into a clean tube and used for fatty acid analysis.

Samples were run on a gas chromatograph (GC) (5890A, Agilent Technologies, Wilmington, DE) in a Supelco-2330 on 100/120 chromosorb WAW column. Samples ran for 20 min on a temperature gradient from 190 to 220°C, being held at the initial temperature 4 min and then increasing in temperature at 4°C min^{-1}. Duplicate FAME samples were prepared from each oil sample extracted. The FAME samples were each run twice on a packed GC column. The oil and FAME from high (40/30°C) and low (28/18°C) temperature seed were extracted and analyzed a third time to test for consistency in the analysis.

Total Nitrogen and Phosphorus Analysis

Seed harvested at maturity were analyzed for total N and P at the University of Florida Forage Evaluation Support Laboratory (Gainesville, FL). Samples were digested using a modification of the aluminum block digestion procedure of Gallaher et al. (1975). Sample weight was 0.25 g, catalyst used was 1.5 g of 9:1 K_2SO_4:$CuSO_4$, and digestion was conducted for at least 4 h at 375°C using 6 mL of H_2SO_4 and 2 mL H_2O_2. The N and P in the digestate were determined by semiautomated colorimetry (Hambleton, 1977).

Carbohydrates

To measure TNC, a modified Nelson-Somogyi test for reducing sugar was used with the addition of amyloglucosidase, α-amylase, and invertase incubation to hydrolyze starch and sucrose (Christiansen, 1982). Approximately 100 mg of ground seed were weighed into a test tube and 4 mL of phosphate buffer were added with 1 mL of α-amylase. The α-amylase had been dialyzed overnight with TRIZMA buffer. This mixture was incubated 90 min at 85°C, and then 5 mL of acetate buffer (pH 4.5) were added with 1 mL of an enzyme mix containing amyloglucosidase and invertase. After an overnight incubation at 50°C, the mixture was filtered and tested with a copper-based color reaction that determined the concentration of reducing sugars in the sample. In this procedure, invertase converts sucrose to glucose and fructose, while α-amylase and amyloglucosidase yield glucose from starch. Total nonstructural carbohydrate analysis measures all carbohydrates present after enzymatic digestion with α-amylase, amyloglucosidase, and invertase. The prepared samples were read on a spectrophotometer (Spectronic 20D, Milton Roy, Rochester, NY) at 540 nm wavelength and TNC was calculated by comparing the optical density readings to those of known concentrations of glucose.

Starch and soluble sugars were determined by weighing 200 mg of ground soybean seed into a test tube containing 3 mL of 80% ethanol. The mixture was held at 80°C for 10 min, centrifuged at ≈12 000 × *g* and the supernatant was removed. This process was repeated twice. The starch pellet was analyzed with the modified Nelson-Somogyi reaction previously described. The soluble sugars were calculated as the difference between the TNC and extracted starch. The proportion of soluble sugars to starch in the TNC was calculated for each temperature treatment.

Statistical Procedures

The Statistical Analysis System Software (SAS Institute, 1993) General Linear Models (GLM) procedure was used for analysis of variance and for regression response vs. temperature. All model effects were considered fixed. Carbon dioxide was used as a classification variable and temperature (TEMP) was a quantitative variable. The model was $X = b_0 + a_1CO_2 + b_1TEMP + b_2TEMP^2$. Because temperature was a quantitative variable, Type I sum of squares was used to determine significance (P level). The above analysis is a conservative statistical test using one mean data point (no replicates) for each treatment combination.

Transcript Analysis

Differential display was performed on total RNA extracted (Chang et al., 1993) from immature seed. Contaminating DNA was removed by incubating the RNA preparation in DNase solution (50 μg RNA, 100 U RNasin, and 5 U RNase-free DNase in 25 μL of 1× reverse transcriptase buffer; all reagents from Promega, Madison, WI) at 37°C for 30 min, then extracting once in acid-buffered phenol (pH 4.0), and once in chloroform, after which the RNA was precipitated with ethanol. Purified RNA was used as template for RT-PCR (using three anchored primers and eight random primers no. 30 through 37 for 24 primer combinations) and products were further processed according to methods described in the RNAIMAGE kit (GenHunter Corp., Nashville, TN) except that RT-PCR products were subjected to electrophoresis through 6% denaturing HR-1000 gel matrix (Genomyx Corporation, Fullerton, CA).

Briefly, DD products were excised, recovered by elution, reamplified by PCR, cloned into pGEM-T (Promega), and sequenced. Sequences were analyzed using the BLAST family of search algorithms (Altschul et al., 1997). RNA gel blots (1.5 μg per lane) were prepared using previously reported methods (Davis et al., 1991) except that RNA was immobilized on Hybond N+ membrane (Amersham Pharmacia Biotech, Piscataway, NJ). Blots were probed with random primer-labeled Gm8 (related to ADR12, an auxin downregulated gene) or Gm19 (related to β-glucosidase), both of which showed a decrease in transcript abundance under elevated temperature. The blot probed with Gm12 [related to an *Arabidopsis thaliana* (L.) Heynh. protein of unknown function as well as soybean expressed sequence tags (ESTs)] was included as a positive control for hybridization; Gm12 transcripts showed no significant regulation by temperature or [CO_2] in other blots (data not shown). Reverse northerns were also performed on Hybond N+ membrane, created by manually spotting 5 to 10 ng of each cDNA arranged in a quartet of spots using a 96-pin colony replicator (Model VP381, V&P Scientific, San Diego, CA). Membranes were probed with ^{32}P-labeled first strand cDNA, prepared from 2 to 5 μg of total RNA, and hybridized and washed the same as northern blots. Spot intensities on the arrays were visualized with a phosphorimager, quantified using the manufacturer's ImageQuant software (Molecular Dynamics, Amersham Pharmacia Biotech, Ithaca, NY), then imported into Microsoft Excel. The magnitude of the induction values for Gm8 were obtained by scaling arrays according to the overall blot means, which resulted in a more conservative estimation of induction than that obtained by scaling according to the overall blot standard deviation, or by scaling relative to Gm12 transcript abundance. Consequently, these are minimum estimates of induction. A total of 18 products were sequenced and assigned GenBank accession numbers (no. CB556156 through CB556173).

RESULTS

Lipid and Fatty Acid Analysis

Total oil concentration was highest at the 32/22°C temperature treatment in both years and decreased at temperatures above 32/22°C (Fig. 2). Oil concentration was linearly related to temperature ($P = 0.042$ in Exp. 1 and $P = 0.007$ in Exp. 2), with a quadratic tendency

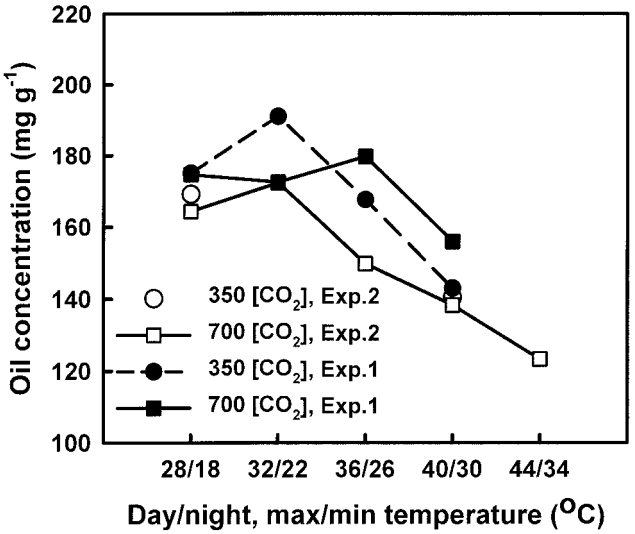

Fig. 2. Mean oil concentration of mature soybean seed in Exp. 1 and Exp. 2 as affected by temperature at 350 or 700 μmol mol^{-1} [CO_2].

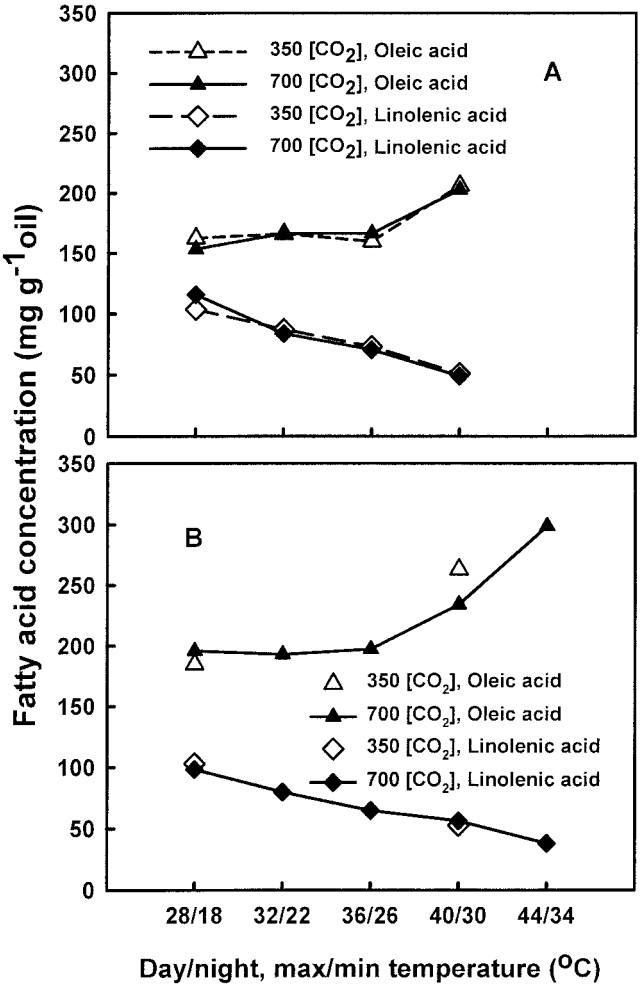

Fig. 3. Changes in oleic and linolenic fatty acid components of soybean seed oil as affected by temperature at 350 or 700 μmol mol^{-1} [CO_2] in Exp. 1 (A) and Exp. 2 (B).

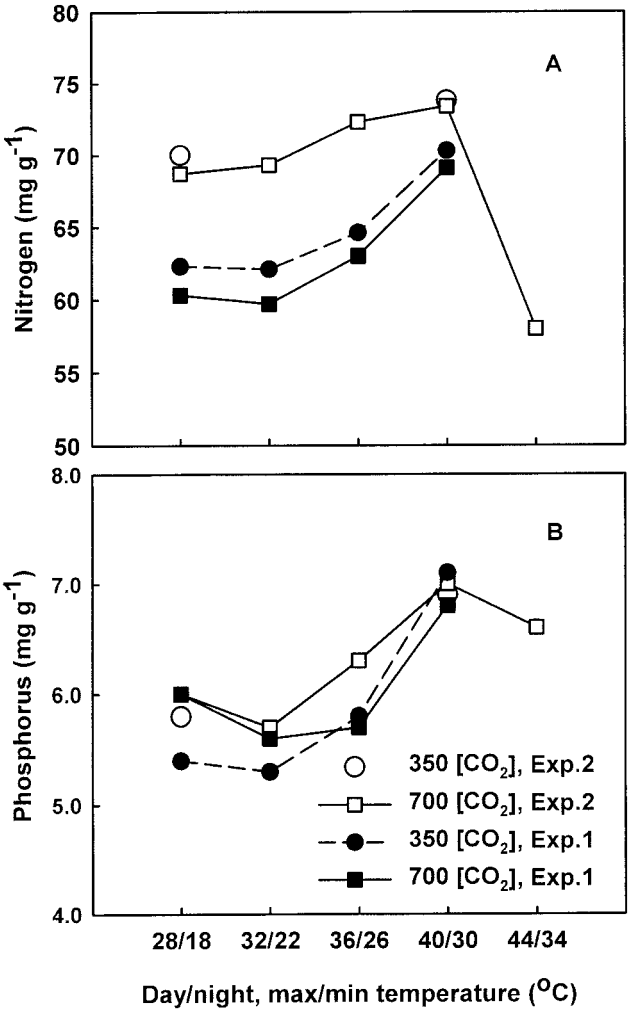

Fig. 4. Concentration of nitrogen (A) and phosphorus (B) in soybean seed as affected by temperature at 350 or 700 μmol mol^{-1} [CO_2].

(P = 0.072 in Exp. 1 and P = 0.141 in Exp. 2). The concentration of oleic acid increased significantly (linear effect, P = 0.011 in Exp. 1 and P = 0.005 in Exp. 2) and that of linolenic acid decreased significantly (linear effect, P = 0.001 in Exp. 1 and P = 0.001 in Exp. 2) with an increase in temperature (Fig. 3). There was a slight quadratic temperature effect on oleic acid in both years (P = 0.069 in Exp. 1 and P = 0.056 in Exp. 2). Temperature responses for these two fatty acids were generally quite comparable in both experiments. Palmitic, stearic, and linoleic acid concentrations, in the extracted seed oil, did not change significantly due to temperature except for a linear temperature effect on palmitic and linoleic acids that occurred only in Exp. 2 at (data not shown). The effects of [CO_2] on total oil and on fatty acid composition were not significant.

Total Nitrogen and Phosphorus Analysis

Nitrogen was reported as elemental N as a percentage of dry matter. The method used was a modification of the standard Kjeldahl procedure; therefore, the value represents total N, including organic (e.g., protein and nonprotein) and inorganic (e.g., nitrate) N. The N concentration of soybean increased as the growth temperature increased up to the 40/30°C treatment and then decreased dramatically (Fig. 4A). There was a tendency (P = 0.070 in Exp. 1) for increased [CO_2] to decrease N concentration in seed, especially at cooler temperatures. The effect of temperature on N concentration was linear (P = 0.001) and quadratic (P = 0.004) in Exp. 1. When the 44/34°C treatment was deleted from statistical analyses, the effect of temperature on N concentration in Exp. 2 was linear (P = 0.028) with no quadratic effect.

The P concentration of harvested seed decreased slightly as the growth temperature increased from 28/18 to the 32/22°C treatment (Fig. 4B). The level of P then increased up until 40/30°C and decreased at the highest temperature treatment, 44/34°C. The effect of temperature was linear (P = 0.001) and quadratic (P = 0.001) on P concentration in Exp. 1. If the 44/34°C treatment was deleted from statistical analyses, the effect of temperature on P concentration was linear (P = 0.021) with a quadratic tendency (P = 0.098) in Exp. 2. There was a slight (P = 0.01) [CO_2] × temperature interaction for P concentration in Exp. 1. There was no significant effect of elevated [CO_2] on either N or P concentration of soybean seed.

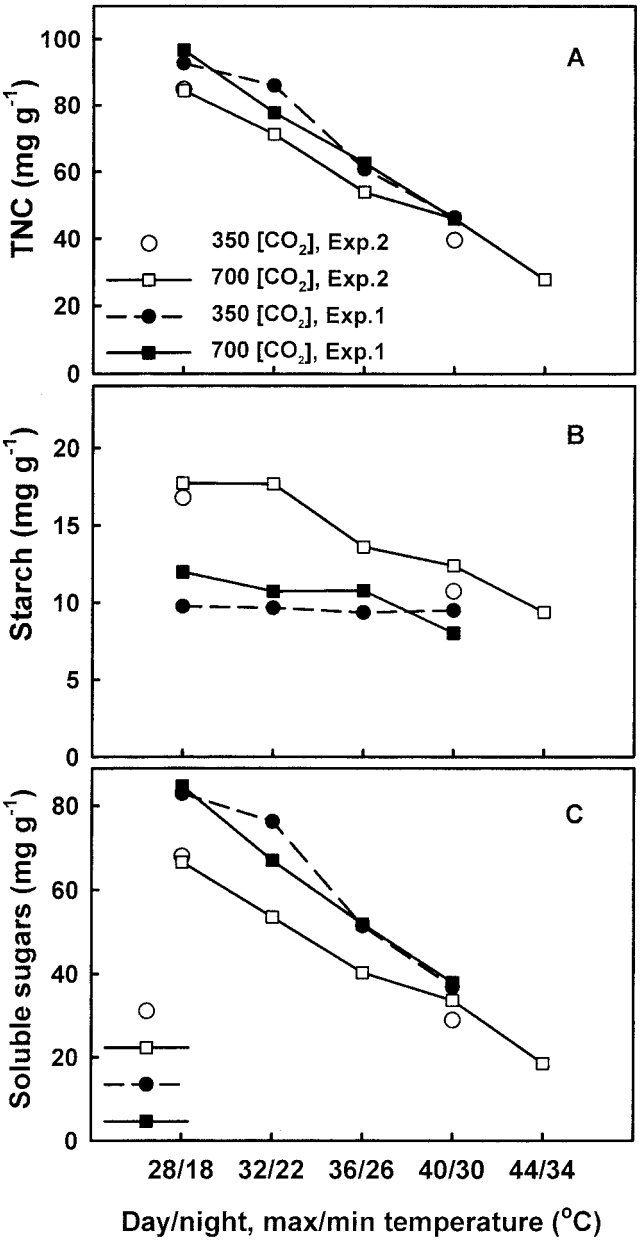

Fig. 5. Total nonstructural carbohydrate concentration (A), starch concentration (B), and soluble sugar concentration (C) of soybean seed as affected by temperature at 350 or 700 μmol mol^{-1} [CO_2].

Carbohydrate Analysis

Soybean TNC was highest, ≈90 mg g^{-1} seed, at the lowest temperature treatment in both years (Fig. 5A). The TNC concentration decreased linearly as the growth temperature increased (P = 0.001 in Exp. 1 and P = 0.001 in Exp. 2). Starch concentration decreased as temperature increased (Fig. 5B). The decrease in starch due to temperature was significant (linear effect, P = 0.048 in Exp. 1 and P = 0.002 in Exp. 2). The concentration of soluble sugars decreased more with increasing temperature than the starch concentration (Fig. 5C) and the temperature effect was linear, P = 0.001 in Exp. 1 and P = 0.001 in Exp. 2. Most of the TNC was in the form of soluble sugars, and the proportion of soluble sugars to starch decreased as the temperature increased.

Differentially Regulated Genes

Eighteen cDNAs were differentially displayed in seed grown at two temperature treatments (40/30 and 28/18°C) and two [CO_2] levels (700 and 350 μmol mol^{-1}). Sequence analysis confirmed that all of the cDNAs were 3′ biased, as would be expected from DD since the anchored primers used in the RT-PCR anneal to the polyA tail. Transcript quantitation by reverse northern analysis revealed that the majority of the genes were regulated moderately, typically threefold or less, and that the differences were not statistically significant in replicated reverse northerns using probes prepared from separate RNA extractions. One notable exception to this was a cDNA (designated Gm8) that hybridized to transcripts that were only detected at the lower temperature (Fig. 6A). Gm8 is 97% identical through 224 bases to the mRNA for ADR12, an auxin down-regulated gene whose translation product, in turn, is 59% identical and 70% similar to the N terminus of plant elongation factor 1 α (e.g., *Nicotiana* AF120093). The Gm8 transcript was 7- to 10-fold more abundant in seed grown at the lower temperature treatments than in seed grown at elevated temperature (Fig. 6B). Another cDNA (designated Gm19) encoded a translation product >80% identical (through 127 amino acids) to plant β-glucosidases (e.g., *Arabidopsis* AF360240). This cDNA was also nearly identical to >10 soybean ESTs identified in various cDNA libraries. The Gm19 transcript regulation was similar to Gm8 but of lesser magnitude. Northern data for a third cDNA (designated Gm12) are included in Fig. 6A as a positive control for probe hybridization, since Gm12 corresponded to a transcript whose abundance was not affected by temperature or by [CO_2].

DISCUSSION

Composition

There were substantial differences in seed composition due to growth temperature for plants grown at temperatures increasing from 28/18 to 44/34°C, and there was no effect due to [CO_2] level (Fig. 2–5). Oil concentration increased with increasing temperature up to 32/22°C, then decreased. These results are similar to the conclusions of both Gibson and Mullen (1996) and Dornbos and Mullen (1992). These data provide information on the elevated temperature range that complements and extends the findings of Piper and Boote (1999), who noted that oil increased with temperature up to a mean temperature of ≈28°C. A quadratic equation gave the best fit for percentage oil vs. temperature in their analysis of 20 soybean cultivars representing 10 maturity groups in 60 locations throughout the USA (Piper and Boote, 1999).

The quadratic effect of oil concentration vs. temperature in the present study (Fig. 2) indicates that 32/22°C is optimum for producing the highest oil concentration in soybean seed. In most soybean-growing regions,

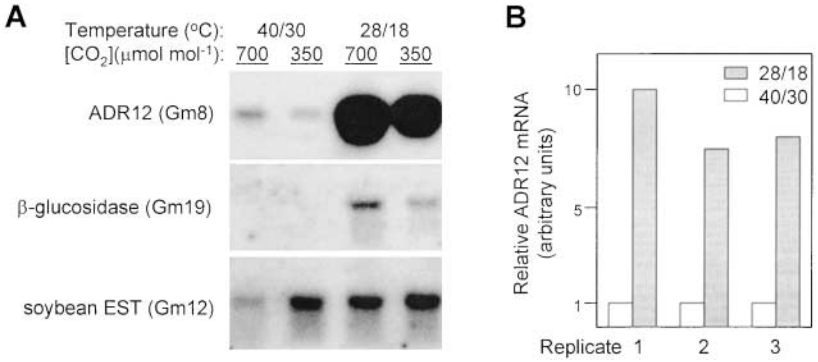

Fig. 6. Steady state mRNA levels of ADR12 and β-glucosidase gene homologs. (A) RNA gel blot data and (B) quantitation of Gm8 transcript abundance by reverse northern analysis.

growth temperatures are below 32/22°C; however, temperatures during the soybean-growing season in the southern USA are at, or slightly higher than, 32/22°C. Therefore, oil concentration at temperatures above 32/22°C would decrease and negatively impact the soybean oil industry in the southern USA, particularly under global climate change conditions such as a 4°C increase in mean growth temperature.

The degree of fatty acid saturation in soybean oil was significantly increased by increasing temperature but there was no effect of elevated [CO_2]. The oleic acid concentration of oil increased and linolenic acid decreased with increasing temperatures from 28/18 to 44/34°C (Fig. 3). Previous soybean studies have shown that oleic acid concentration increased with increasing temperatures while linoleic acid decreased (Carver et al., 1986; Rennie and Tanner, 1989; Gibson and Mullen, 1996; Rebetzke et al., 1996). The temperatures in these studies were not as high, and our study confirms this trend continues up to 44/34°C. Changes in the fatty acid composition, such as concentration of oleic acid, are associated with nutritional aspects as well as storage longevity of soybean oil (O'Byrne, 1995; Mounts et al., 1988). Oleic acid is more stable because it is monounsaturated, and with only one double bond it is less susceptible to oxidation than linolenic acid, which has three double bonds.

Nitrogen or crude protein concentration increased with temperature to 40/30°C, but above 40/30°C, protein concentration decreased sharply (Fig. 4A). Piper and Boote (1999) reported lowest protein concentration at 22°C (mean), increasing as temperature increased to 28°C (mean). The present study shows that this increasing trend continued from our lowest temperature 28/18°C (23°C mean) up to 40/30°C (35°C mean). Protein concentration in soybean seed that developed at a range of mean temperatures from 15.5 to 30.5°C was relatively stable but increased significantly at the highest temperature (Wolf et al., 1982). Seed from soybean plants exposed to daytime air temperatures of 35°C during seedfill contained four percentage points more protein than those exposed to air temperatures of 29°C (Dornbos and Mullen, 1992) and seed protein concentration increased linearly when grown at temperatures from 28 to 33°C (Gibson and Mullen, 1996). We hypothesize that increasing protein concentration with decreasing oil may actually be a mathematical side effect caused by increased temperature effects to decrease oil and TNC concentration, since the fractions must add to 1.0. For example, assume a reference seed of 100 mg seed mass with 20 mg oil and 40 mg crude protein. If the amount of oil decreases to 18 mg because of elevated temperature effects, and the protein remains at 40 mg, then seed mass becomes 98 mg, and oil concentration is now 184 mg g^{-1} while protein is now 408 mg g^{-1}. Soybean protein concentration is negatively correlated to seed oil (Krober and Cartter, 1962; Burton, 1987; Dornbos and Mullen, 1992; Piper and Boote, 1999).

At 44/34°C, decreased seed protein concentration could possibly be caused by limited N_2 fixation, which may be reduced at temperatures above 40/30°C (Gibson, 1971, 1975). Since there was no fertilizer N added to the soil during the experiment, the decrease in N concentration at 44/34°C could be related to inhibition of biological N_2 fixation at that temperature. Hungria and Vargas (2000) stress the vulnerability of rhizobial strains to high temperature.

The pattern of P concentration in seed was similar to that for N concentration. (Fig. 4B). About 50% of P in soybean is in phytate, which is the calcium-magnesium-potassium salt of inositol hexaphosphoric acid, commonly known as phytic acid. Phytate is often located within the protein bodies, thus it is logical that P increases with N concentration (Liu, 1997).

Total nonstructural carbohydrates decreased continuously as temperatures increased (Fig. 5A), indicating that available assimilates are increasingly converted to lipid, protein, or structural components. Lower carbohydrate levels in seed with increasing temperature would suggest assimilate availability or transfer into seed was limited, as hypothesized by Thorne (1982). Phloem unloading from the seed coat vascular bundles into the apoplast surrounding the cotyledon cells has been shown to be inhibited under anaerobic conditions (Thorne, 1982). We hypothesize that O_2 availability to seed may be limiting as temperature increases, because O_2 solubility is less and respiratory demand is higher as temperature increases. Several authors have shown a decrease in seed weight mediated by decreased O_2 partial pressures (pO_2) (Quebedeaux and Hardy, 1975; Sinclair et al., 1987), a similar trend to the effects of higher temperature.

The data for both levels of CO_2 are shown in the graphs even though the effects of [CO_2] were not significant. Heagle et al. (1998) found a significant effect of elevated CO_2 on soybean seed oil in cultivars Essex, Holladay, and NK6955. The oleic acid concentration was positively affected by [CO_2], and the effect of cultivar was also highly significant. There was no effect of CO_2 on protein concentration in the studies of Heagle et al. (1998). A recent meta-analysis of 79 reports on plant reproduction under elevated CO_2 by Jablonski et al. (2002) showed that legume seed N concentration was not affected by growth at elevated CO_2, but that seed N concentration declined significantly in most nonlegumes. Allen et al. (1998) reported no significant effect of [CO_2] on seed TNC when soybean (cv. Bragg) was grown at six levels of CO_2 from 160 to 990 μmol mol^{-1}.

Transcript Abundance

Examination of transcripts by DD from immature seed was intended to help identify molecular processes that may be affected by climate change. Examination of transcript abundance shifts may assist in analyzing how and why temperature treatments affected seed characteristics, by sampling from the transcript pools of seeds from the high and low temperature treatments at both levels of [CO_2]. Temperature effects on transcript abundance were significant and more dramatic than [CO_2] effects (Fig. 6A), as we did not identify cDNAs that were specifically [CO_2]-regulated. This is consistent with the biochemical data indicating that temperature effects were significant while there were no measurable [CO_2] effects. These results are not unexpected, given that a chronic treatment of twofold higher [CO_2] causes more seed to be added (Baker et al., 1989) and is not a physiologically acute stress when compared with a chronic treatment of 12°C temperature increase.

The Gm8 cDNA is similar to ADR12, a transcript that is rapidly and significantly downregulated by exogenous auxin treatment (Datta et al., 1993). Interestingly, ADR12 is also rapidly downregulated in the roots of soybean during the early stages of cyst nematode feeding site formation (Hermsmeier et al., 1998). We have no evidence to suggest a role for auxin in down-regulating ADR12 in soybean seeds grown at elevated temperature, although auxin concentration is known to increase during normal seed development in *Phaseolus coccineus* L. (Picciarelli et al., 2001). It appears that Gm8 downregulation may simply reflect the dramatic shift in developmental trajectory that occurs when seeds develop under elevated temperature; however, these seeds were sampled at similar days after initiation of pod growth (Fig. 1). Because Gm8 is regulated so dramatically, it appears to be a promising expression marker for future research directed at understanding underlying mechanisms.

The Gm19 cDNA is similar to plant β-glucosidase, and its transcript accumulated in the low temperature treatment, but was not detected at high temperature. In plants, β-glucosidase activities have been identified that utilize isoflavone glucosides, cyanogen glucosides, or phytohormone glucosides as substrates, hydrolyzing them into aglycone forms (Esen, 1993). These enzymes may be located in any of several different cellular compartments. Because it is not possible at present to identify the substrate for the enzyme encoded by Gm19, it is difficult to speculate on its specific role. However, it is reasonable to infer from the regulation of Gm19 that the normal cellular balance of glycosylated and nonglycosylated compounds may be impaired in the cells of a seed developing in high temperature. This could affect general metabolite trafficking between the organelles and/or processes in which the glycosylated and nonglycosylated compounds play distinct cellular roles. The apparent loss of β-glucosidase transcript at elevated temperature suggests that glycosylated products would accumulate under these conditions, thus metabolic profiling under low and high temperatures would be a potential tool for identifying substrate(s) for this enzyme product.

We used DD for these experiments, which served as an initial survey for transcript differences and provided positive controls for future experiments. Comprehensive screens for shifts in transcript abundance are now feasible by using microarrays, which permit simultaneous monitoring of thousands of different transcripts (Shoemaker et al., 2002) and thus should provide a more complete picture of how the soybean seed transcriptome responds to environmental stress.

In summary, higher temperatures significantly affected seed composition, with the effects of elevated [CO_2] being comparatively small and insignificant. Our findings suggest minimal concern for rising [CO_2] effects on composition and edible quality of soybean seed. However, depending on the current temperature of a region, elevated temperature, if it occurs, will have considerable impact on seed composition, and will be accompanied by changes in transcript abundance. Additional study is necessary to understand the biochemical bases for these phenomena.

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REFERENCES

Allen, L.H., Jr., and K.J. Boote. 2000. Crop ecosystem responses to climatic change: Soybean. p. 133–160. *In* K.R. Reddy and H.F. Hodges (ed.) Climate change and global crop productivity. CABI Publ., New York.

Allen, L.H., Jr., E.C. Bisbal, and K.J. Boote. 1998. Nonstructural carbohydrates of soybean plants grown in subambient and superambient levels of CO_2. Photosynth. Res. 56:143–155.

Altschul, S.F., T.L. Madden, A.A. Schaffer, J.H. Zhang, Z. Zhang, W. Miller, and D.J. Lipman. 1997. Gapped BLAST and PSI-BLAST: A

new generation of protein database search programs. Nucleic Acids Res. 25:3389–3402.

Augustin, J., and B.P. Klein. 1985. Nutrient composition of raw, cooked, canned, and sprouted legumes. p. 187–217. *In* R.H. Matthews (ed.) Legumes: Chemistry, technology and human nutrition. Marcel Dekker, New York.

Baker, J.T., and L.H. Allen, Jr. 1993. Contrasting species responses to CO_2 and temperature: Rice, soybean and citrus. Vegetatio 104(105):239–260.

Baker, J.T., L.H. Allen, Jr., K.J. Boote, P. Jones, and J.W. Jones. 1989. Response of soybean to air temperature and carbon dioxide concentration. Crop Sci. 29:98–105.

Burton, J.W. 1987. Quantitative genetics: Results relevant to soybean breeding. p. 211–247. *In* J.R. Wilcox (ed.) Soybeans: Improvement, production, and uses. Agron. Monogr. 16. 2nd ed. ASA, CSSA, and SSSA, Madison, WI.

Carver, B.F., J.W. Burton, T.E. Carter, Jr., and R.F. Wilson. 1986. Response to environmental variation of soybean lines selected for altered unsaturated fatty acid composition. Crop Sci. 26:1176–1180.

Chang, S., J. Puryear, and J. Cairney. 1993. A simple and efficient method for isolating RNA from pine trees. Plant Mol. Biol. Rep. 11:113–116.

Christiansen, S. 1982. Energy reserves and agronomic characteristics of four limpograsses for Florida's flatwoods. Ph.D. diss. Univ. of Florida, Gainesville, FL.

Curry, R.B., J.W. Jones, K.J. Boote, R.M. Peart, L.H. Allen, Jr., and N.B. Pickering. 1995. Response of soybean to predicted climate change in the USA. p. 163–182. *In* Climate change and agriculture: Analysis of potential international impacts. Spec. Publ. 59. ASA, Madison, WI.

Datta, N., P.R. LaFayette, P.A. Kroner, R.T. Nagao, and J.L. Key. 1993. Isolation and characterization of three families of auxin downregulated cDNA clones. Plant Mol. Biol. 21:859–869.

Davis, J.M., H.R.G. Clarke, H.D. Bradshaw, and M.P. Gordon. 1991. *Populus* chitinase genes—Structure, organization, and similarity of translated sequences to herbaceous plant chitinases. Plant Mol. Biol. 17:631–639.

Dornbos, D.L., and R.E. Mullen. 1992. Soybean seed protein and oil contents and fatty acid composition adjustments by drought and temperature. J. Am. Oil Chem. Soc. 69:228–231.

Esen, A. 1993. β-Glucosidases: Overview Chapter 1. *In* A. Esen. (ed.) β-Glucosidases: biochemistry and molecular biology. Am. Chemical Soc., Washington, DC.

Fehr, W.R., and C.E. Caviness. 1977. Stages of soybean development. Cooperative Ext. Service Spec. Rep. 80. Iowa State Univ., Ames, IA.

Gallaher, R.N., C.O. Weldon, and J.G. Futral. 1975. An aluminum block digester for plant and soil analysis. Soil Sci. Soc. Am. Proc. 39:803–806.

Gibson, A.H. 1971. Factors in the physical and biological environment affecting nodulation and nitrogen fixation in legumes. p. 139–152. *In* T.A. Lie and E.G. Mulder (ed.) Plant and Soil special volume. Martinus Nijoft, The Hague, The Netherlands.

Gibson, A.H. 1975. Recovery and compensation by nodulated legumes to environmental stress. p. 385–445. *In* P.S. Nutman (ed.) Nitrogen fixation in plants. Cambridge Univ. Press, Cambridge.

Gibson, L.R., and R.E. Mullen. 1996. Soybean seed composition under high day and night growth temperatures. J. Am. Oil Chem. Soc. 73:733–737.

Hambleton, L.G. 1977. Semi-automated method for simultaneous determination of phosphorus, calcium, and crude protein in animal feeds. J. Assoc. Off. Anal. Chem. 60:845–852.

Heagle, A.S., J.E. Miller, and W.A. Pursley. 1998. Influence of ozone stress on soybean response to carbon dioxide enrichment: III. Yield and seed quality. Crop Sci. 38:128–134.

Heppard, E.P., A.J. Kinney, K.L. Stecca, and G.H. Miao. 1996. Developmental and growth temperature regulation of two different microsomal ω-6 desaturase genes in soybeans. Plant Physiol. 110: 311–319.

Hermsmeier, D., M. Mazarei, and T.J. Baum. 1998. Differential display analysis of the early compatible interaction between soybean and the soybean cyst nematode. Mol. Plant–Microbe Interact. 11: 1258–1263.

Honeycutt, R.J., J.W. Burton, R.G. Palmer, and R.C. Shoemaker. 1989. Association of major seed components with a shriveled-seed trait in soybean. Crop Sci. 29:804–809.

Hungria, M., and M.A.T. Vargas. 2000. Environmental factors affecting N_2 fixation in grain legumes in the tropics, with an emphasis on Brazil. Field Crops Res. 65:151–164.

Hymowitz, T., F.I. Collins, J. Panczer, and W.M. Walker. 1972. Relationship between the content of oil, protein, and sugar in soybean seed. Agron. J. 64:613–616.

Intergovernmental Panel on Climate Change. 1995. Radiative forcing of climate change. p. 349–416. *In* J.T. Houghton et al. (ed.) Climate change, 1995: The science of climate change. Cambridge Univ. Press, Cambridge.

Jablonski, L.M., X. Wang, and P.S. Curtis. 2002. Plant reproduction under elevated CO_2 conditions: A meta-analysis of reports on 79 crop and wild species. New Phytol. 156:9–26.

Keeling, C.D., T.P. Whorf, M. Wahlen, and J. Van der Plicht. 1995. Interannual extremes in the rate of rising of atmospheric carbon dioxide since 1980. Nature (London) 375:660–670.

Krober, O.A., and J.L. Cartter. 1962. Quantitative interrelations of protein and nonprotein constituents of soybeans. Crop Sci. 2: 171–172.

Liang, P., and A.B. Pardee. 1992. Differential display of eukaryotic messenger RNA by means of the polymerase chain reaction. Science (Washington, DC) 257:967–971.

Liu, K. 1997. Soybeans: Chemistry, technology and utilization. Chapman and Hall, New York.

Maxwell, R.J., and W.N. Marmer. 1983. Systematic protocol for the accumulation of fatty acid data from multiple tissue samples: Tissue handling, lipid extraction and class separation and capillary gas chromatographic analysis. Lipids 18:453–459.

Messina, M. 1997. Soyfoods: Their role in disease prevention and treatment. Ch. 10. *In* K. Liu (ed.) Soybeans: Chemistry, technology and utilization. Chapman and Hall, New York.

Mounts, T.L., K. Warner, G.R. List, R. Kleiman, W.R. Fehr, E.G. Hammond, and J.R. Wilcox. 1988. Effect of altered fatty acid composition on soybean oil stability. J. Am. Oil Chem. Soc. 65:624–628.

O'Byrne, D.M.J. 1995. The effects of a low fat diet high in monounsaturated fatty acids on serum lipids, apolipoproteins, and lipoproteins in postmenopausal women with hypercholesteremia. Ph.D. diss. Univ. of Florida, Gainesville, FL.

Pan, D. 1996. Soybean responses to elevated temperature and doubled CO_2. Ph.D. diss. Univ. of Florida, Gainesville, FL.

Picciarelli, P., N. Ceccarelli, F. Paolicchi, and G. Calistri. 2001. Endogenous auxins and embryogenesis in *Phaseolus coccineus.* Aust. J. Plant Physiol. 28:73–78.

Pickering, N.B., L.H. Allen, Jr., S.L. Albrecht, P. Jones, J.W. Jones, and J.T. Baker. 1994. Environmental plant chambers: Control and measurement using CR-10T dataloggers. p. 29–35. *In* D.G. Watson et al. (ed.) Computers in agriculture 1994. Am. Soc. Agric. Engineers, St. Joseph, MI.

Piper, E.L., and K.J. Boote. 1999. Temperature and cultivar effects on soybean seed oil and protein concentrations. J. Am. Oil Chem. Soc. 76:1233–1241.

Quebedeaux, B., and R.F.W. Hardy. 1975. Reproductive growth and dry matter production of *Glycine max* (L.) Merr. in response to oxygen concentration. Plant Physiol. 55:102–107.

Rebetzke, G.J., V.R. Pantalone, J.W. Burton, B.F. Carver, and R.F. Wilson. 1996. Phenotypic variation for saturated fatty acid content in soybean. Euphytica 91:289–295.

Rennie, B.D., and J.W. Tanner. 1989. Fatty acid composition of oil from soybean seeds grown at extreme temperatures. J. Am. Oil Chem. Soc. 66:1622–1624.

SAS Institute. 1993. SAS user's guide. Version 6. 2nd ed. SAS Inst., Cary, NC.

Sasser, M. 1990. Identification of bacteria by gas chromatography of cellular fatty acids. MIDI Tech. note no. 101. MIDI, Newark, DE.

Shoemaker, R., P. Keim, L. Vodkin, E. Retzel, S.W. Clifton, R. Wa-

terston, D. Smoller, V. Coryell, A. Khanna, J. Erpelding, X.W. Gai, V. Brendel, C. Raph-Schmidt, E.G. Shoop, C.J. Vielweber, M. Schmatz, D. Pape, Y. Bowers, B. Theising, J. Martin, M. Dante, T. Wylie, and C. Granger. 2002. A compilation of soybean ESTs: Generation and analysis. Genome 45:329–338.

Sinclair, T.R., J.P. Ward, and C.A. Randall. 1987. Soybean seed growth in response to long-term exposures to differing oxygen partial pressures. Plant Physiol. 83:467–468.

Thomas, J.M.G. 2001. Impact of elevated temperature and carbon dioxide on development and composition of soybean seed. Ph.D. diss. Univ. of Florida, Gainesville, FL.

Thorne, J.H. 1982. Temperature and oxygen effects on ^{14}C-photosynthate unloading and accumulation in developing soybean seeds. Plant Physiol. 69:48–53.

Wolf, R.B., J.F. Cavins, R. Kleiman, and L.T. Black. 1982. Effect of temperature on soybean seed constituents: Oil, protein, moisture, fatty acids, amino acids and sugars. J. Am. Oil Chem. Soc. 59: 230–232.